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Development of a novel, non-invasive diagnostic test for
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PRINCIPAL INVESTIGATOR:
Robert Alexander Gardiner

CONTRACTING ORGANIZATION:
University of Queensland
Brisbane, Australia QLD 4072
AUSTRALIA

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14. ABSTRACT To examine ejaculate in the diagnosis of prostate cancer, initially we confirmed that seminal fluid was comparable with prostatic fluid collected in urine following prostatic massage as a source for analysis for expression of the non-coding RNA PCA3 in relation to PSA RNA. For a total of 158 cases, we identified a sensitivity of 63%, a specificity of 72.5% (94% negative predictive value). Based on extensive molecular profiling of malignant and benign prostatic tissue in which a combination of 3 highly-discriminating markers detected 100% of cancers, we examined these in ejaculate and post-ejaculate urines. Results were then analysed in conjunction with serum prostate specific antigen (PSA) levels, clinically the primary indication for diagnostic biopsies currently. Our best results were obtained using a combination of ejaculate PSA and hepsin in conjunction with serum PSA which provided an 82% sensitivity and a 92% specificity for a negative predictability of 89% for 44 patients. The study is ongoing.					
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INTRODUCTION

Prostate cancer (**PCa**) is the second most common life threatening cancer in Australian men with 1 in 11 being diagnosed in their lifetimes. Diagnosis is typically through serum measurements of prostate specific antigen (**PSA**) a protein normally secreted specifically by prostate epithelial cells to form a component of ejaculate, to identify males at-risk of harbouring PCa. Definitive diagnosis and clinical staging require a digital rectal exam (**DRE**) and transrectal ultrasound (**TRUS**) biopsies.

PSA & PCa diagnosis: PSA testing with PCa diagnosis and treatment are growth areas¹. Based on Medical Benefits (MBS) item numbers, PSA testing for PCa in Australia increased from 492,147 in 2001-02 to 698,828 in 2005-06¹ and shows no signs of slowing. The age-standard incidence rate for PCa rose sharply following introduction of PSA testing, peaking in 1994 then falling until 1998, followed by another sharp rise which is ongoing. Separations for radical prostatectomy (**RP**), the commonest treatment for PCa in Australia increased from 2,007 in 2000-2001 to 4,925 in 2005-6 with 54% of these procedures performed on the 60-69 age group & 33.4% on men aged 50-59 years¹ in 2004-05.

Diagnostic use of PSA: PSA is not cancer-specific and there is no threshold level providing a high sensitivity and specificity with a continuum of risk for all PSA values². A raised serum PSA so often commits men to the invasive and imprecise procedure of transrectal ultrasound (**TRUS**) guided biopsies. The diagnostic 'strike' rate with TRUS biopsies depends on the population tested with many reports citing a 25% detection rate³. The number of biopsy cores taken is also relevant with sextant biopsies missing ~25% of tumours and 12 cores missing up to ~10%⁴. A further indictment is the disparity between TRUS and RP with the former undercalling pathology⁵.

The PSA-TRUS biopsy approach has been optimised over the years so that a quantum change is required to improve detection. We promoted the use of prostatic fluid focusing on seminal fluid and post ejaculate urine while others have used prostatic fluid obtained by prostatic massage to measure for DD3/PCA3 and other markers. We have not adopted the latter approach since prostatic fluid obtained in this manner is likely to be derived from the posterior part of the gland exclusively with concerns that vigorous massage will facilitate tumour cell dissemination and metastasis formation. Ejaculate contains the natural exocrine effluent of the prostate which is extruded by contraction of the extensive network of smooth muscle in the gland at the time of ejaculation. This project was designed to further our experience with ejaculate and post-ejaculate urine using molecular profiling to identify the presence of cancer cells in the prostate.

BODY OF REPORT

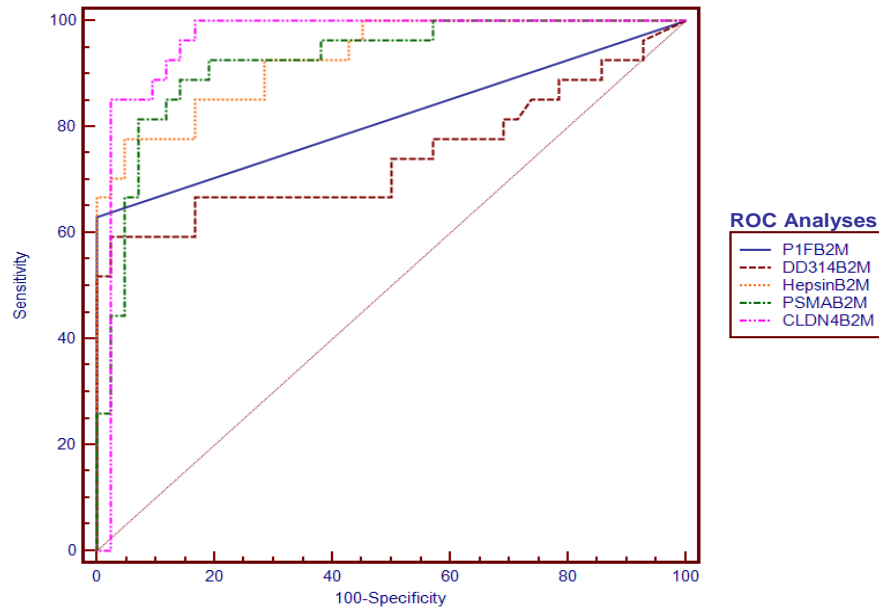
This study explores molecular profiling of enriched prostatic cells from ejaculate for detecting prostate cancer. Instead of continuing to try to refine even further the current 2-step diagnostic process using the PSA blood test to identify potential prostate cancer patients followed by biopsies with traditional histopathology to diagnose the minority of patients actually harbouring the malignancy, this study directs its focus to the prostate cancer cells themselves in a “one-step” assay using molecular profiling. Our strategy involves sampling the prostate by three different methods, a) by traditional TRUS biopsy b) by collecting prostate cells secreted in ejaculate, and (c) by collecting cells secreted in post-ejaculate urines for study and then collating clinical data from histopathology from biopsies with novel biomarker analyses of the biological fluid samples. We aim to develop a routine, rapid, non-invasive method of sampling the prostate, and detecting prostate cancer cells accurately.

Molecular Profiling of Tissues: To identify molecular PCa markers for use in combination with PCA3 we undertook molecular profiling of benign and malignant prostatic tissue and identified a list of candidate markers⁶⁻⁸. Four biomarkers, UDP-N-Acetyl- α -D-galactosamine transferase (**GalNAc-T3**), PSMA, Hepsin and DD3/PCA3, which, in combination, distinguished prostate cancer from Benign Prostate Hyperplasia (**BPH**) were overexpressed in PCa tissues by microarray analysis, confirmed by quantitative Real-time PCR and shown, immunohistochemically to be localised to prostate epithelial cells with higher expression in malignant cells.

PCR and shown, immunohistochemically to be localised to prostate epithelial cells with higher expression in malignant cells. Real-time quantitative PCR analysis across 21 PCa and 34 BPH tissues showed 4.6 fold overexpression of GalNAc-T3 ($p=0.005$). The non-coding mRNA (DD3/PCA3) was overexpressed 140 fold ($p=0.007$) in the cancer samples compared with BPH tissues. Hepsin was overexpressed 21 fold ($p=0.049$); while the overexpression for PSMA was 66 fold ($p=0.047$). When the gene expression data for these 4 biomarkers was combined in a logistic regression model, a predictive index was obtained which distinguished 100% of the PCa samples from all of the BPH samples demonstrating a powerful new approach to diagnosing PCa by molecular profiling⁷. Subsequently, we found the Claudin 4 transcript, encoding a cellular adhesion molecule, was superior to GalNAc-T3 in this role so we introduced Claudin 4 as a replacement 4th marker. Notably, Claudin 4 was more highly expressed in low grade (Gleason 6) primary PCas compared with high grade (Gleason >7) cancers raising the potential for this marker to indicate low-risk PCa, unlikely to progress⁸.

The Receiver Operating Curve (ROC) was used to assist in deciding the cutpoint to maximize sensitivity and specificity. The ROC analysis for the top 4 markers in relation to β 2-microglobulin (as an indicator of the presence of RNA) is shown in **Figure 1** immediately below.

Figure 1: Prostatic tissue studies



Nucleic-Acid Sequence Based Amplification (NASBA) analyses: Having pioneered the use of prostatic fluid in the early diagnosis of PCa^{9, 10}, we applied our findings from our tissue analyses to disaggregated cells in prostatic fluid. However, before examining multiple markers, we evaluated the suitability of the RNA approach by examining DD3/PCA3 RT-PCR in relation to PSA RT-PCR (to indicate the presence of prostatic cells) in ejaculate to confirm that at least comparable results were provided compared with that obtained by others using DD3/PCA3 RT-PCR of RNA from urine following prostatic massage. In this exercise, we emulated the approach used by others employing the NASBA technique which excludes the possibility of evaluating other markers.

Ejaculate results were very comparable with post-prostatic massage urines (**Table 1** immediately below).

Table 1 Study	Sensitivity	Specificity	Negative Predictive Values	Number
Hessels et al, Eur Urol 2003 ¹¹	67%	83%	90%	108
Fradet et al, Urology 2004 ¹²	66%	74%	84%	517
Tinzl et al, Eur Urol 2004 ¹³	82%	76%	87%	158
van Gils et al, Clin Cancer Res 2007 ¹⁴	65%	66%	80%	534
Van Gils et al, Prostate 2007 ¹⁵	65%	82%	80%	67
Our results with ejaculate	63%	72.5%	94%	158

Table 1: Mean sensitivity & specificity for prostatic massage results^{11,12,13,14,15} were 69% & 76%, respectively, compared with 63% & 72.5% for ejaculate.

Rationale for post-DRE urines: Following these findings, we then proceeded to examine ejaculate and post-ejaculate urines for the 4 markers identified as most discriminating in our tissue analyses. However, since all other investigators had used post-prostatic massage prostatic fluid, we decided that we should also collect prostatic fluid obtained in urine following Digital Rectal Examination (**DRE**) (as opposed to following massage) in addition to prostatic fluid obtained from ejaculate and urine immediately following ejaculation, for our assays. This decision was particularly prompted by the published results of the multi-centre study (table number?) by Fradet et al (2004¹²) and the decision by Bostwick Laboratories provide a test commercially that assays for PCA3/DD3 RNA (<http://www.bostwicklaboratories.com/>) from prostatic cells in urine immediately following prostatic massage^{11,13}. These assays use NASBA (Nucleic-Acid Sequence Based Amplification¹⁶) to amplify the PCA3/DD3 from samples of RNA.

DoD-imposed disruption to study: After submitting our first annual report to the US Department of Defense, we were contacted by the Human Research Protections Office (HRPO), Office of Research Protections U.S. Army Medical Research and Material Command because of their concern that we were including post-DRE urines in our study. Despite lengthy, though helpful and accommodating interactions with Debra de Paul for the Human Protection Office, we had to dispense with including this source of prostatic fluid for our analyses because we could not meet the requirements stipulated by the DoD in terms of their requirements for informed consent.

As a result, clinical recruitment for this study was halted from 9 February 2006 and restarted only on 28 April 2007 because of the above issue and other requirements of the Human Research Protections Office (HRPO), Office of Research Protections U.S. Army Medical Research and Material Command with which we inadvertently had not complied. (We had initially commenced recruiting as planned in year one on the basis of an approval from Dr Mishra in his email of 19 November 2004). In the period during which we could not collect specimens from patients, we reverted to amplifying with standard PCR from the start (as per our application for funding) rather than using NASBA. The reason for reverting to RT-PCR

was to ensure that comparable RNA sequences were available for detection of multiple markers from each patient, the NASBA method being suited for single marker detection from each RNA sample. Although the disruption to specimen collection caused a serious loss of momentum to this study, we have continued to undertake this research and the current status is outlined below. **Current approach:** We have obtained and analysed seminal fluid from 48 patients who underwent transrectal ultrasound-guided (TRUS) biopsies, 46 of whom provided ejaculates and 48 post-ejaculate urines. All patients had at least 12 cores taken at the time of their TRUS biopsies.

Because these analyses are RNA-based and RNA degradation is time-dependent in the enzyme-rich environment that is ejaculate, time is of the essence. Consequently, patients delivered specimens as soon as possible following producing them so that they could be processed within 2 hours in our laboratory.

Protocol: Ejaculate specimens (diluted in Hanks buffer) were layered immediately onto a percoll column and centrifuged at 2200 rpm for 30 min. The supernatant on top of the mononuclear cell layer was removed, aliquoted and stored at -80°C until further processing. The mononuclear cells were collected and washed twice with PBS. Trizol was added and the samples frozen at -80°C. Once a number of samples had been collected, the batch was processed and RNA isolated and cleaned using the RNeasy kit. Urine samples were centrifuged and then washed twice with PBS prior to storage.

When a reasonable number of specimens had been collected, stored specimens were then thawed, RNA extracted and expanded (Genisphere RNA expansion kit) to be stored as cDNA until analysis. Single stranded cDNA was then tested for β_2 -microglobulin.

Table 2: Findings for the 46 ejaculate & 48 post-ejaculate urines for all markers

Specimen	Marker	Cancer status	n	1 st quartile	Median	3 rd quartile	p-value*
Ejaculate	CLDN4 (normalised to PSA)	neg	27	.29	2.25	5.30	0.40
		pos	19	.26	1.43	3.29	
Ejaculate	PSMA (normalised to PSA)	neg	27	1.15	3.35	44.04	0.47
		pos	19	1.80	6.25	54.71	
Ejaculate	Hepsin (normalised to PSA)	neg	27	.13	.71	10.11	0.20
		pos	19	.10	.22	3.69	
Ejaculate	DD3/PCA3 (normalised to PSA)	neg	27	.42	1.58	6.41	0.01
		pos	19	2.56	9.71	22.07	
Post-ejaculate urine	CLDN4 (normalised to PSA)	neg	27	1.11	5.35	28.24	0.11
		pos	21	.26	1.59	9.86	
Post-ejaculate urine	PSMA (normalised to PSA)	neg	27	1.21	11.28	55.15	0.38
		pos	21	1.05	6.17	29.24	
Post-ejaculate urine	Hepsin (normalised to PSA)	neg	27	.83	2.94	12.61	0.12
		pos	21	.32	1.63	5.42	
Post-ejaculate urine	DD3/PCA3 (normalised to PSA)	neg	27	.62	2.47	61.27	0.34
		pos	21	.73	1.79	10.23	

* Mann-Whitney test on ranked data as the data have strong positive skew

(Notice that ejaculate DD3/PCA3 does not provide complete discrimination: the top 25% of the non cancer group have higher ejaculate DD3/PCA3 readings than the bottom 25% in the cancer group). PSA was used as an indicator of prostatic RNA.

Findings from multiple marker studies of ejaculate

To determine the commonality between the specimens and for commonalities within each specimen type, results were normalised and log transformed. In addition, cross correlations were normalised to serum PSA and log transformed. This showed that the different biomarkers within the same specimen type had more in common than the same biomarkers from the two different specimen types.

Transformations

Since in clinical practice a combination of predictive findings are integrated in both informal and formal decision-making considerations, all markers were normalised to PSA to determine skewness and kurtosis in the raw data. Logarithm transformations were found to provide the best correction for the skew and kurtosis in the data. (Various power transformations were tested but are not shown here: 2nd, 3rd and 4th roots, inverse squared and inverse cubed).

Approach to statistical analysis

It is known that assay results can vary from kit to kit. To address this control markers are incorporated in each run and data are expressed relative to these control values. In this study we have both PSA and B₂M available to use as control markers. The literature and our investigations suggested that PSA is the most appropriate control marker. Therefore, all data reported in this study has been normalised to PSA (i.e. expressed as a ratio of the PSA measure).

Descriptive analyses were used to reveal the shapes of the distributions. Many of the variables display strong positive skew – see for example PSA serum results in the following chart **Figure 2** (immediately below).

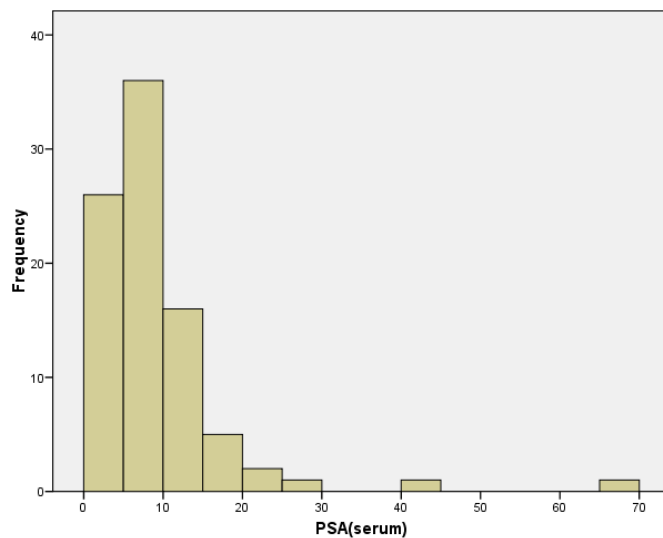


Figure 2:
Serum PSA

Strongly skewed distributions invite either non-parametric analyses or data transformations prior to parametric analyses. We have employed both approaches in our analyses and achieved complimentary results. Most of the results shown in this report are derived from transformed data. Logarithm transformations were found to provide the best correction for

the skew and kurtosis in the data. (Various power transformations were tested but are not shown here: 2nd, 3rd and 4th roots, inverse squared and inverse cubed.) The log transformed PSA data shown in **Figure 3** (immediately below) provides one confirmation of the effectiveness of the transformation in removing skew.

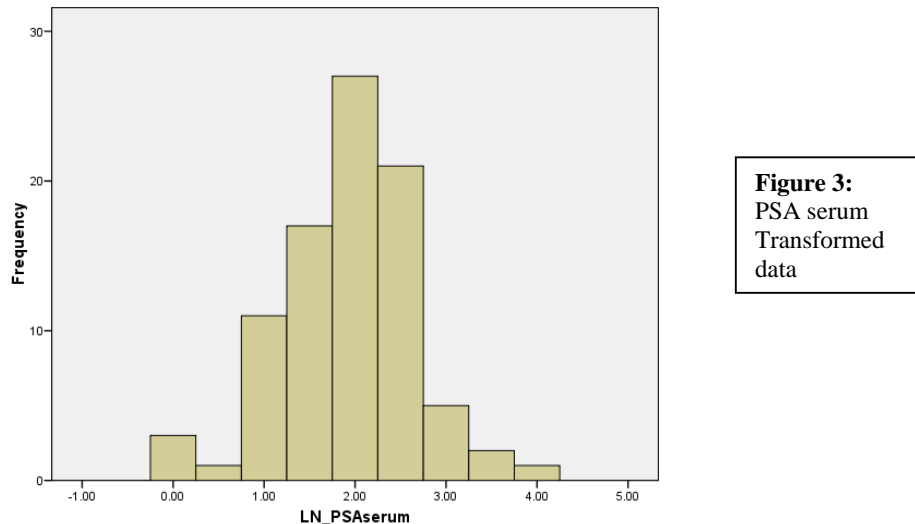


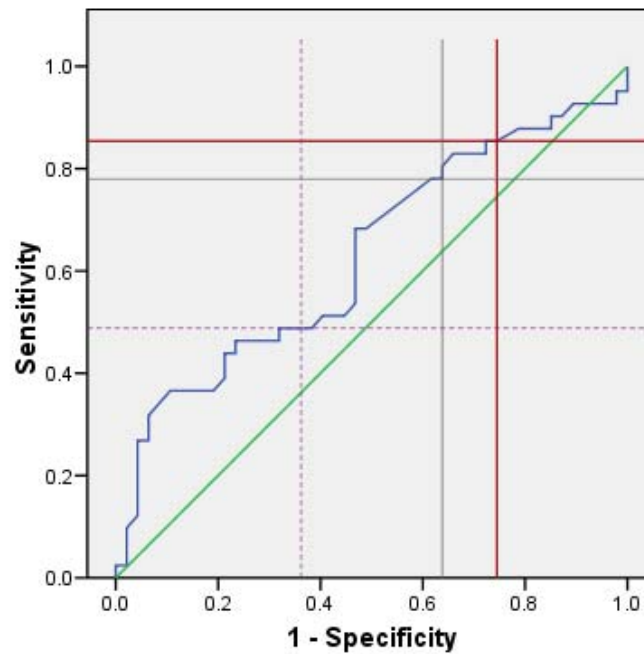
Table 3 (immediately below) gives skewness and kurtosis statistics before and after the log transformation. (Skewness is completely resolved, kurtosis is still about 2 standard errors above zero – therefore there is still some statistical evidence of kurtosis).

Table 3	N	Skewness		Kurtosis	
	Statistic	Statistic	Std. Error	Statistic	Std. Error
PSA (serum)	88	4.348	.257	25.907	.508
LN_PSA serum	88	-.124	.257	1.196	.508
Valid N (listwise)	88				

One of the aims of the analysis was to investigate the ability of the various markers to differentiate between the cancer group and a control group. Following the literature, the tools we have used to summarise the markers ability to differentiate the two groups are sensitivity, specificity and associated receiver operating curves. Sensitivity is the proportion of people with the disease who are correctly identified using the predictive biomarkers. Specificity is the proportion of people without the disease who are correctly ruled out using the biomarkers. As the biomarkers normalised scores range across a continuum, the sensitivity and specificity of the biomarkers can be varied by varying the diagnostic threshold value. The receiver operating curve (ROC) maps the sensitivities and specificities obtained for all possible threshold values. For example, Figure 4 shows the ROC curve produced by using varying values of PSA serum as indicators of prostate cancer in our data set. The blue line is the ROC. The location of the 4ng/ml cutpoint is marked on the ROC in red. This cutpoint gives a relatively high sensitivity (i.e. most of those with cancer have PSA serum > 4ng/ml) but low specificity (i.e.

many of those without cancer also have PSA serum >4 ng/ml). The grey line shows sensitivity and specificity for the 5ng/ml cutpoint and the purple dotted line indicating the 8ng/ml cutpoint. The higher the cutpoint, the smaller the proportion of cancer patients above it (i.e. lower sensitivity) and the lower proportion of control patients above it (i.e. improved specificity).

Figure 4: ROC analysis serum PSA



Diagonal segments are produced by ties.

In this particular example when PSA serum ≥ 4 ng/ml, 85% of men with cancer would be correctly categorised (sensitivity) and 26% of men without cancer would be correctly categorised (specificity). Of those detected by the PSA serum test, 50% would actually have cancer (positive predictive value). Of those not detected by the test, 67% did not have cancer in their TRUS biopsies (negative predictive power).

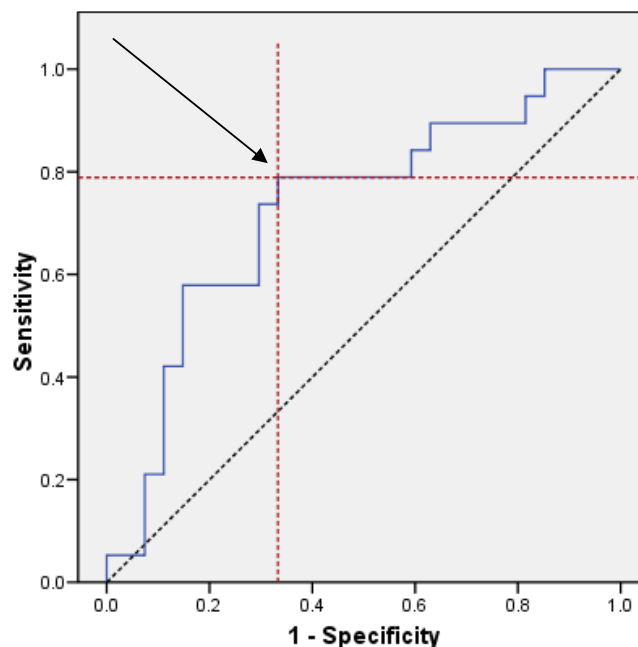
The green line (at 45 degrees) shows the ROC which would have been obtained if the biomarker was completely unrelated to the disease (had no predictive power). The area between the blue and green line shows the predictive characteristics of the biomarker. The higher the blue line is above the green, the greater the predictive power, and the greater the range of cutpoints the blue line remains high, the more robust the biomarker is to slight variations in threshold values. We used logistic regression to model the combined discriminatory power of two or more biomarker used in conjunction. Predicted values from the logistic model are presented using ROC curves in the same way as above.

Combining specimen types

The data set contains observations from two types of specimens viz. 46 Ejaculate specimens and 48 Urine specimens. We used a logistic regression model with cancer as the outcome and specimen type as a predictor variable. The first marker included in the model is ejaculate DD3 as it showed the strongest discriminatory power in the bivariate comparisons above.

The ROC curve was used to assist in deciding the cutpoint to maximise sensitivity and specificity.

Figure 5: ROC analysis ejaculate DD3/PSA



The point marked corresponds to a predicted value $p=0.4050$ or in $(DD3/PSA)=0.8463$ or $(DD3/PSA)=2.33$ and delivered sensitivity=79% and specificity=67% on this data set.

Applying the single cutpoint we now get the logistic regression equation:

$$\text{logit}(p) = -1.322 + 1.950(s/_{DD3_{cut}})$$

Where:

1. The variable is a statistically significant predictor of cancer (Chi-square=7.48, df=1, p=0.006)
2. As $\exp(1.95)=7.03$, the odds of cancer are 7 times higher among those in the higher ejaculate_DD3 category than those in the lower category

We then examined whether adding any of the other markers would improve discriminatory power.

Stepwise analyses (not shown) revealed that ejaculate hepsin showed some statistical evidence of predictive power ($p=0.06$) over the information in ejaculate DD3. (Urine hepsin ($p=0.72$) was not a statistically significant predictor but was followed up as Hepsin has shown discriminatory power in other circumstances viz. the original tissue samples). The charts show the information added by ejaculate hepsin and urine hepsin respectively (all normalised to PSA with logarithm transformations).

Figure 6: ROC analysis ejac PCA3/DD3 & ejaculate Hepsin

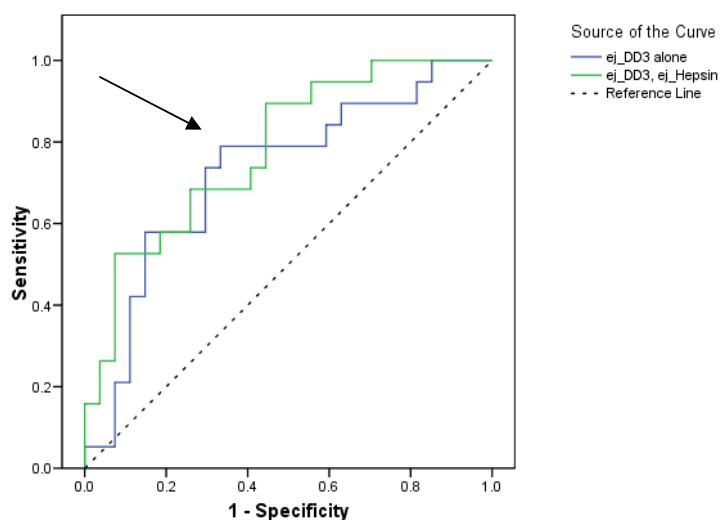
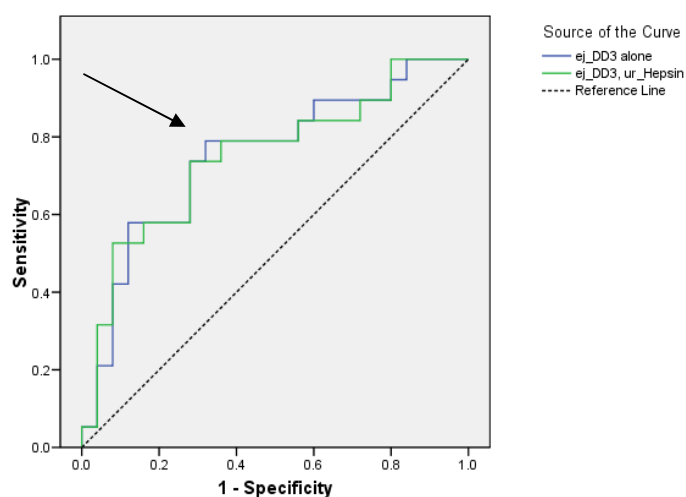


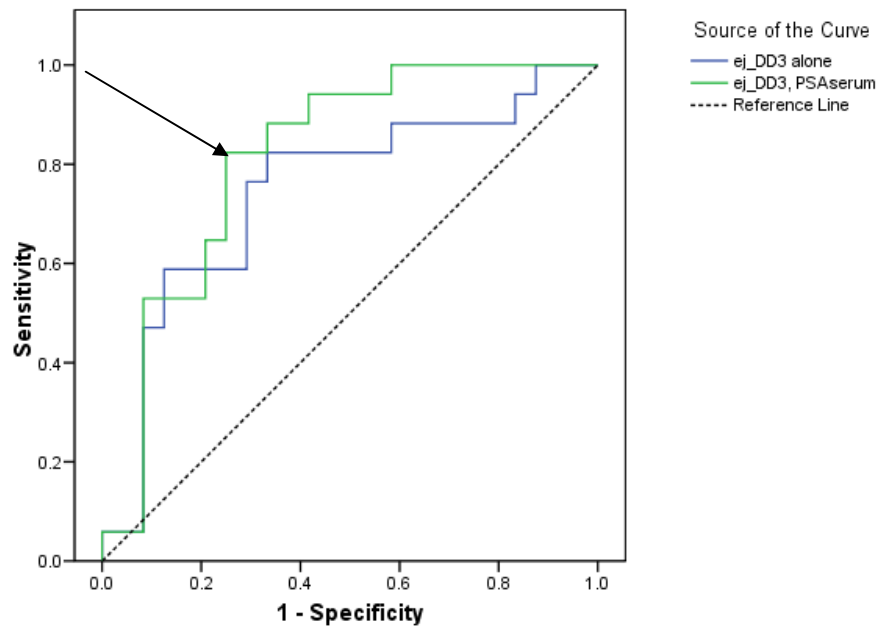
Figure 7: ROC analysis ejaculate PCA3/ DD3 & Urinary Hepsin



Adding the ejaculate hepsin seemed to make predictions worse in the key area of maximal sensitivity and specificity.

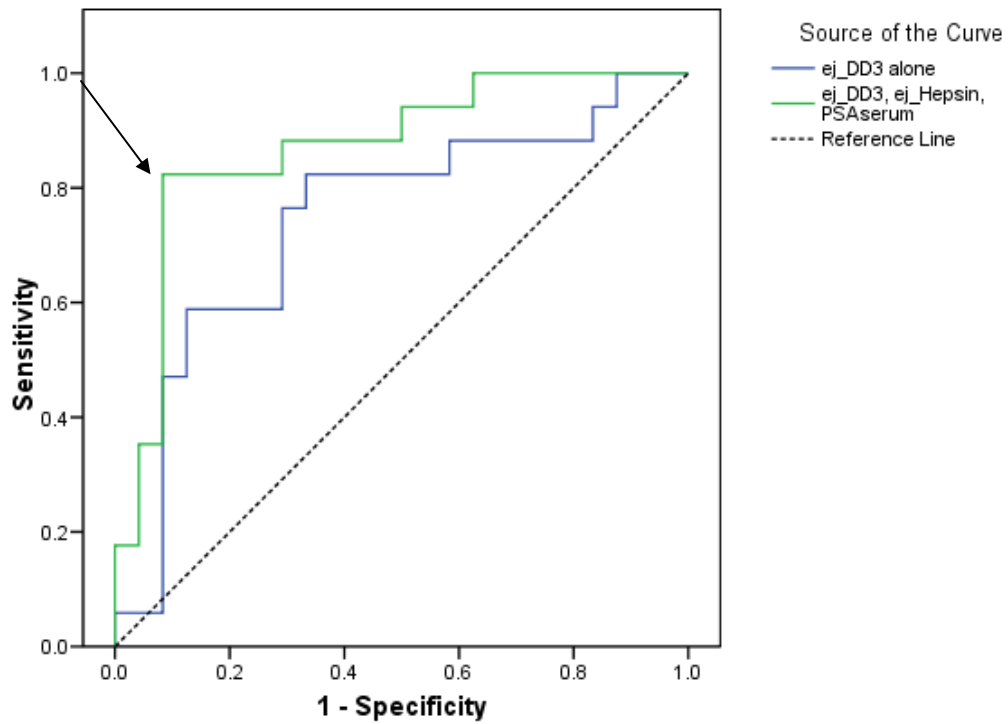
PCA3/DD3 ($p=0.010$) and serum PSA ($p=0.06$) showed simultaneous evidence of predictive power and inclusion of serum PSA would be predicted to improve sensitivity from 79% to 82% and improve specificity from 67% to 75%. Perhaps more importantly, including PSA serum increased the range of potential cutpoints with a high sensitivity and specificity (making the test more robust, less reactive to minor variations in measurements, etc).

Figure 8: ROC analysis ejac PCA3/DD3 & serum PSA



The best results were obtained when all 3 of these predictors were included in a single model. Each delivered some statistically significant predictive power, over and above the information in the other 2 variables (ej_PCA3/DD3 has $p=0.007$, ej_Hepsin has $p=0.035$, serum PSA has $p=0.042$). The highest achievable discriminatory power was sensitivity of 82% and specificity of 92%.

Figure 9: ROC analysis ejac DD3, ejac Hepsin & serum PSA



Two possible explanations arise: either the information in the 3 markers was complementary or adding the extra parameter to the model on a relatively small sample size is overfitting of the model to the data.

The equivalent ROC analysis with ejaculate PCA3/DD3, urine hepsin and PSA serum provided a maximum sensitivity of 82% and specificity of 73%. The addition of urine Hepsin and serum PSA made the test more robust (i.e. more accurate over a larger range of possible cutpoints) but did not have much impact on the estimated peak achievable accuracy.

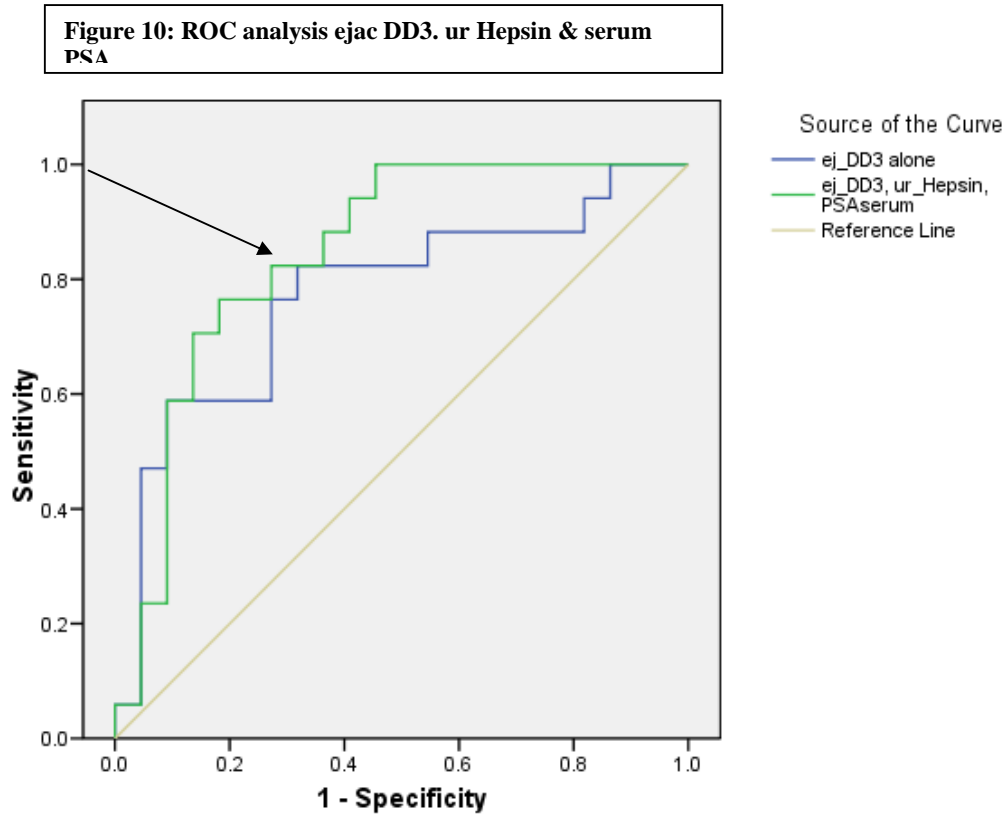


Table 4: PCA3 alone in ejaculate & ejaculate PCA3 & hepsin with serum PSA

Study	Sensitivity	Specificity	Negative Predictive value	Number
Our NASBA DD3/PCA3 ejaculate results	63%	72.5%	94%	158
Our combined serum PSA, ejaculate DD3/PCA3 & ejaculate hepsin results	82%	92%	89%	44

KEY RESEARCH ACCOMPLISHMENTS:

- Confirmation that ejaculate is a comparable with post-prostatic massage urine as a source of prostatic fluid for the diagnosis of prostate cancer using PCA3 RT-PCR
- Identified a profile of multiple discriminating molecular markers that permits a 100% detection rate for prostate cancer in tissue specimens
- Demonstrated that a combination of the markers PCA3 and Hepsin in ejaculate together with serum PSA is most effective in discriminating patients with a biopsy diagnosis of prostate cancer from those whose biopsies are negative for cancer

REPORTABLE OUTCOMES

PUBLICATIONS EMANATING FROM THE GRANT

Clarke R, Zhao Z, Guo A-Y, Roper K, Teng L, Fang Z-M, Samaratunga H, Lavin MF, Gardiner RA. Increased complexity of the prostate-specific gene PCA3: implications for the sensitive detection of prostate cancer. Plos One under review

Landers KA, Samaratunga H, Teng L, Burger MJ, Scells B, Lavin MF, Gardiner RA. Identification of Metastatic Markers for Prostate Cancer. BJ Cancer, 2008; 99:491-501

Teng L, Buck M, Yameen Z, Scells B, Samaratunga MLTH, Yaxley J, Stening S, MF Lavin, Gardiner RA. PCA3 and Claudin 4 in prostate tissue and prostatic fluid. BJUI, 2008; 101(suppl 1): Abstract 124 (ASM Urological Society of Australia and New Zealand)

Gardiner RA, Burger MJ, Landers KA, Scells B, Samaratunga MLTH, Yaxley J, Stening S, MF Lavin. Multiple molecular markers in the diagnosis of prostate cancer from prostatic fluid. BJUI, 2007; 99(suppl 2): Abstract 2 (ASM Urological Society of Australia and New Zealand)

CONCLUSIONS:

The findings from our preliminary work endorsing our operative hypothesis that a combination of markers rather than any one by itself is required to detect all prostate cancers is vindicated. Although the combination of PCA3 and Hepsin, based on molecular profiling of tissues, appears to be most discriminating, addition of the most pertinent clinical indicator for diagnostic biopsy, serum PSA, to the analysis improves the results considerably. With the numbers accrued to date in this ongoing study, a combination of DD3/PCA3 in ejaculate and Hepsin in ejaculate with serum PSA permits much better detection of prostate cancer than serum PSA alone or serum PSA plus PCA3/DD3.

It is clear that the combination of markers reported here will not detect every prostate cancer with a high specificity. However, the fact that the diagnostic reference, TRUS biopsy, remains less than perfect even when >12 or more cores are obtained for histological examination, indicates that the approach pursued in this study appears to be nearing that of TRUS biopsy in detecting prostate cancer. Consequently, we are confident that with accrual of further numbers, the results from this work will provide a significant advance in the identification of those patients referred for consideration of TRUS biopsy who harbour prostate cancer.

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APPENDICES

LIST OF PERSONNEL WHO RECEIVED PAYMENT FROM THE GRANT

Miss L Teng	BSc
Mrs B Scells	Enrolled Nurse
Dr Z Yameen	BSc, PhD
Dr MJ Burger	BSc, PhD

ABBREVIATIONS

PCa	Prostate cancer
PSA	Prostate specific antigen
DRE	Digital rectal examination
TRUS	Transrectal ultrasound
GalNAc-T3	UDP- <i>N</i> -Acetyl- α -D-galactosamine transferase
BPH	Benign prostatic hyperplasia
ROC	Receiver operating characteristic
NASBA	Nucleic-acid Sequence Based amplification
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
cDNA	Complementary deoxy-ribonucleic acid

